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Noncoding RNA of Glutamine Synthetase I Modulates Antibiotic Production in *Streptomyces coelicolor* A3(2)^{∇‡}

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Overexpression of antisense chromosomal *cis*-encoded noncoding RNAs (ncRNAs) in glutamine synthetase I resulted in a decrease in growth, protein synthesis, and antibiotic production in *Streptomyces coelicolor*. In addition, we predicted 3,597 *cis*-encoded ncRNAs and validated 13 of them experimentally, including several ncRNAs that are differentially expressed in bacterial hormone-defective mutants.

In many diverse organisms, noncoding RNA (ncRNAs) have been identified as major regulators of translation efficiency and message stability (9, 23). Recent screens for ncRNAs in human predict approximately 30,000 structured RNAs (22), and many have been characterized as having a variety of regulatory functions and thus are not simply transcriptional by-products or “transcriptional noise” (2, 5). However, the number of ncRNAs identified in bacteria is lagging behind those found in eukaryotes. Since the first publication of a fully sequenced bacterial genome, a number of genomewide computational screens for ncRNAs in microorganisms have been conducted, predicting 50 to 2,000 ncRNAs (11). ncRNAs are grouped into *cis*-encoded or *trans*-encoded classifications, depending on whether the ncRNA structure is located in the transcribed sequence of its target or found at a distance, for instance, in an intergenic region. The former type of ncRNA is reported mainly in bacterial plasmids, whereas the latter class accounts for most of the ncRNAs reported in bacterial chromosomes. These *trans*-encoded ncRNAs require helper proteins such as Hfq to bind to the target mRNA. They can either degrade or stabilize the target mRNA, and they are supposed to be involved in the regulation of metabolic pathways due to environmental change or stress responses (23). Recently, *cis*- and *trans*-encoded ncRNAs have been identified in mycobacterium, and the function of a *trans*-encoded ncRNA has been demonstrated (1). Several groups or researchers (12, 18, 20) have predicted and partially validated the existence of ncRNAs

in *Streptomyces*, a genus of Gram-positive soil bacteria with high biotechnological relevance due to their diverse secondary metabolite production, including the production of a large variety of antibiotics of commercial importance. However, in all of these cases where *trans*-encoded ncRNAs were identified, their function (if any) or mode of action remained completely unknown.

In the model streptomycete *Streptomyces coelicolor*, no functional analysis of ncRNA has been conducted, nor are there any homologues to the main components of the RNA silencing system in the eukaryote, or the main ncRNA processing enzymes in prokaryotes, apart from homologues to enzymes, which are able to degrade double-stranded RNA. To explore a potential role of ncRNA in *S. coelicolor*, we first focused on the *glnA* gene (SCO2198), which encodes for glutamine synthetase I (GSI) (6), a key regulatory target of central nitrogen metabolism and major player in the link of nitrogen assimilation to antibiotic production. An intragenic ncRNA in *glnA* called “*cnc2198.1*” was predicted (Fig. 1). The *cnc2198.1* ncRNA was detected in early exponential phase for the wild-type and two signaling molecule-impaired strains tested by reverse transcription-PCR (RT-PCR) and tagged-primer-specific quantitative RT-PCR (qRT-PCR; data not shown) (14). Primer-specific cDNA synthesis was performed with tagged primer to retrotranscribe the antisense transcript and then used as a template for qRT-PCR analysis (see Table S2 and other supplementary material). We went on to test whether this ncRNA would also be functional, despite the absence of any previously characterized RNA-binding proteins involved in the processing of

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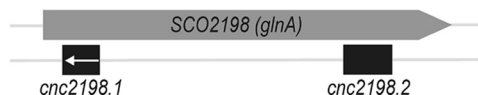


FIG. 1. Location of ncRNA loci in SCO2198. SCO2198 (genomic coordinates 2364905 to 2366314) is represented in gray, while the predicted 121-bp ncRNA locus *cnc2198.1* (genomic coordinates 2364929 to 2365049) and 161-bp ncRNA locus *cnc2198.2* (genomic coordinates 2365728 to 2365888) are shown in black on the opposite DNA strand. The locus *cnc2198.2* was not analyzed further. The white arrow represents the 88-bp fragment cloned to overexpress the antisense RNA.

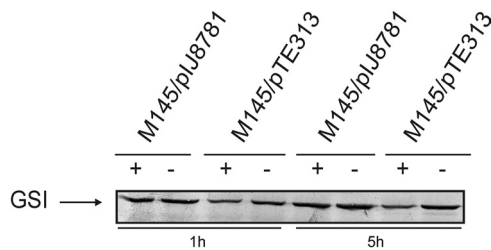


FIG. 2. Western blot analysis of GSI. Protein levels were analyzed by Western hybridization with anti-GSI specific antibodies at 1 and 5 h after induction of *cnc2198.1as* (M145/pTE313) expression (+) or addition of DMSO (-) in M145/pIJ8781 or M145/pTE313.

ncRNA identified in eukaryotes or prokaryotes. An antisense 88-bp fragment that overlaps with *cnc2198.1* (*cnc2198.1as*; 120 bp) was cloned into the multicopy plasmid pIJ8781 under the control of the thiostrepton-inducible promoter *tipAp* (see the supplemental material). The resulting plasmid pTE313, as well as the vector pIJ8781, was introduced into *S. coelicolor* M145. The two strains were grown to an optical density at 450 nm (OD_{450}) of 0.7 in SMM (19), and the expression of *cnc2189.1as* was induced with thiostrepton in dimethyl sulfoxide (DMSO; 5 μ g/ml). Pure DMSO was used as a negative control. When *cnc2198.1as* expression was induced, GSI signal detected by

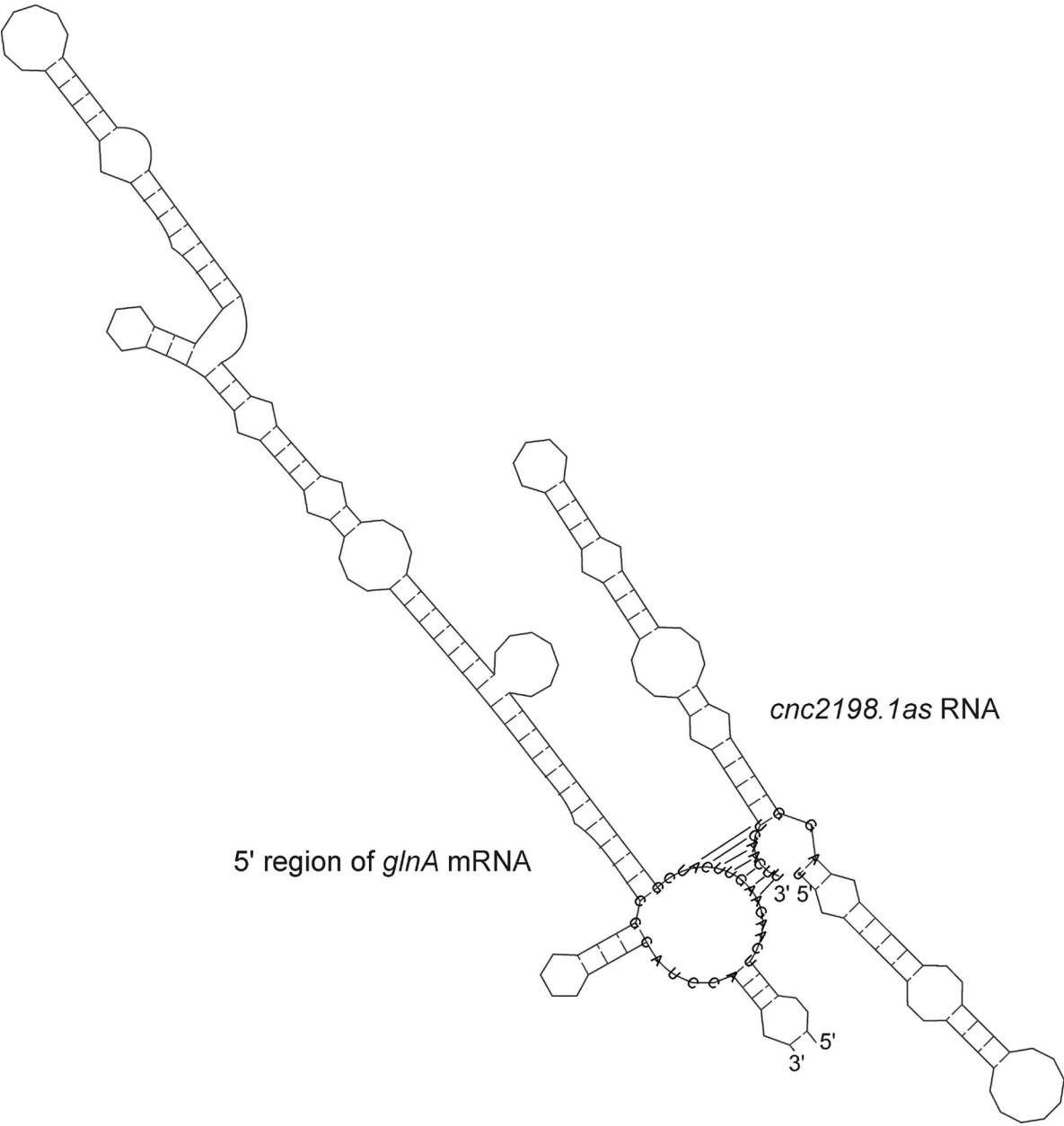


FIG. 3. Predicted secondary structure of *glnA* mRNA and *cnc2198.1as* RNA. The web-based RNAfold software was used to predict the secondary structure of the *glnA* and *cnc2198.1as* transcripts. The single-stranded regions in the structures are indicated as the most likely nucleotides to be involved in the interaction between the two RNA molecules; however, the interaction between ncRNA and mRNA could also occur before the secondary structure formation or involve rearrangements in the secondary structure, for instance, by RNA helicases.

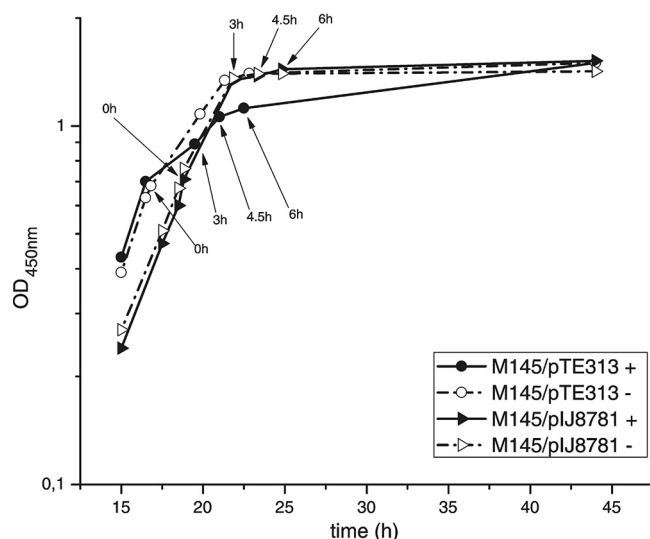


FIG. 4. Growth curve of M145/pIJ8781 and M145/pTE313. M145/pIJ8781 and M145/pTE313 growth in liquid SMM supplemented with apramycin (1 μ g/ml) was determined. Samples induced for *cnc2198.las* expression by thiostrepton (+) are depicted as black lines with black circles (M145/pTE313), induced empty vector control (M145/pIJ8781) is depicted as a black line with black triangles, and negative control samples with DMSO (–) are depicted as dashed lines with white circles (M145/pTE313) or triangles (M145/pIJ8781). Various time points for antibiotic measurements are indicated by arrows.

Western analysis with a GSI antibody (dilution, 3:10,000) using 100 μ g of total protein extract, decreased to 72% \pm 9% and 63% \pm 6% at 1 and 5 h, respectively, in comparison to M145 harboring the vector alone (Fig. 2). (The values are averages of three independent growth curves.) This shows that *cnc2198.las* indeed affects protein expression levels of its target gene *glnA* by perhaps interacting with the few nucleotides of *glnA* mRNA (Fig. 3) inducing the unfolding and the complementary annealing of the two transcripts that would lead to a stall of translation and/or degradation, possibly through a mechanism independent of the classical ncRNA processing enzymes.

This molecular effect also had important and wide-ranging regulatory consequences at the phenotypic level. Not only did growth decrease (Fig. 4), but there was also a substantial effect on antibiotic production, the major regulated physiological process of *Streptomyces*. The red pigmented antibiotic undecylprodigiosin production of M145/pTE313 decreased 4-fold after induction of *cnc2198.las* expression compared to the negative control and \sim 3.5-fold compared to the level seen in M145/pIJ8781 when measured as reported (19) (Fig. 5).

To explore the general relevance of ncRNAs in *Streptomyces*, we also conducted a computational genomewide prediction of ncRNAs by using RNAz v1.01 on the 54% of the genome of *S. coelicolor* that aligned with *S. avermitilis* as reported previously in yeast (17; see also the supplemental material). The presence of ncRNA was predicted where the RNA classification confidence value was >0.5 . Of the 3,895 ncRNA elements predicted, 3,597 overlapped with 2,965 annotated coding sequences, and the remaining 298 were located in the intergenic regions. The predicted ncRNAs included all tRNAs and rRNAs annotated in RFAM and TIGR (see Table S1 in the supplemental material). Our predictions also overlapped

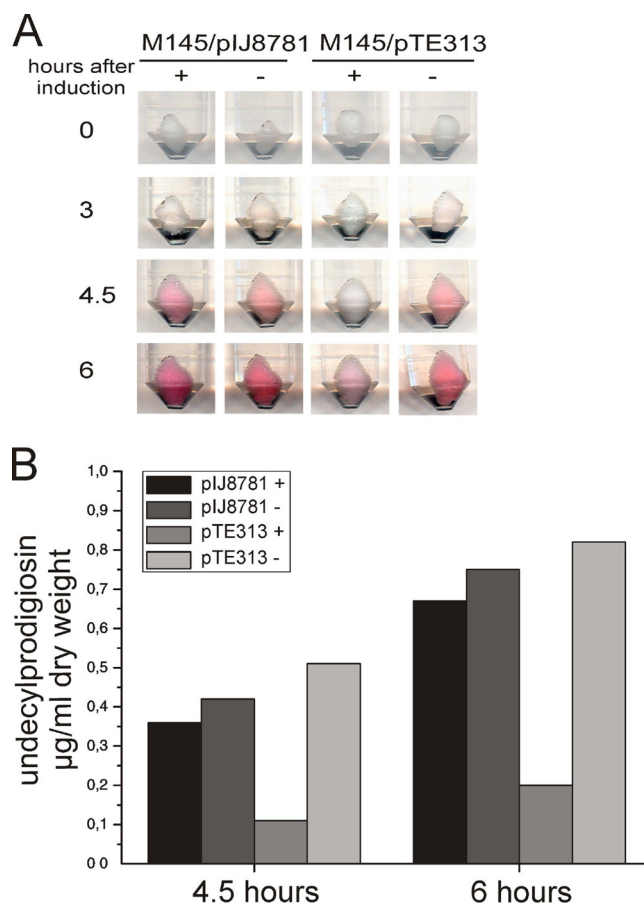


FIG. 5. Antibiotic production by M145/pIJ8781 and M145/pTE313. (A) Cell pellets of M145/pIJ8781 or M145/pTE313 treated with thiostrepton (+) or DMSO (–) were collected at the time of induction (0 h) and after 3, 4.5, or 6 h. (B) The same cells were then measured for the pigmented antibiotic, undecylprodigiosin, as indicated in the bar graph.

to a large extent with those found in previous studies: 28 out of 32 predictions by Pánek et al. (12) and 55 of 114 predictions by Swiercz et al. (18) were confirmed.

Total RNA was extracted as reported previously (19), and ncRNA transcripts were analyzed by primer-specific qRT-PCR (see Table S2 and other supplemental material). For each transcript, the signal was normalized to the specific transcriptional background. Signals above the negative control *cnc2966* (no ncRNA predicted) were considered validated. Of the 18 predicted *cis*-encoded ncRNA elements that were analyzed, 13 were shown to have antisense ncRNA present either in the wild type or in two signaling molecule (γ -butyrolactone) mutants (Table 1). These 18 ncRNAs correspond to those genes which were differentially expressed in a γ -butyrolactone mutant (E. Takano et al., unpublished data). The signaling molecules are bacterial hormones that regulate antibiotic production in *Streptomyces*. Interestingly, two ncRNAs (*cnc3669.2* and *cnc3893.2*) were detected only in the two signaling molecule impaired mutants and were not present in the wild type. This, for the first time, suggests that these signaling molecules are involved in the regulation of ncRNA expression in *S. coelicolor*.

This is the first report to elucidate a *cis*-encoded antisense

TABLE 1. Confirmation of predicted antisense ncRNAs

ncRNA ^a	Predicted antisense ncRNAs ^b		
	M145	M751	M752
<i>cnc2966</i>	– (3.06 ± 1.02)	– (5.09 ± 0.37)	– (1.04 ± 0.13)
<i>cnc0981</i>	+ (18.21)	+ (66.04)	+ (12.53)
<i>cnc0982</i>	+ (35.97)	+ (247.95)	+ (55.31)
<i>cnc2819</i>	+ (230.86)	+ (NA)	+ (NA)
<i>cnc3005(5-7)</i>	+ (NA)	+ (NA)	+ (NA)
<i>cnc3005.5</i>	+ (NA)	+ (NA)	+ (NA)
<i>cnc3005(1-2)</i>	– (NA)	– (NA)	– (NA)
<i>cnc3669.1</i>	+ (56.95)	+ (692.54)	+ (93.13)
<i>cnc3669.2</i>	– (3.70)	+ (150.26)	+ (17.27)
<i>cnc3893.2</i>	– (1.54)	+ (7.11)	+ (2.11)
<i>cnc4296</i>	– (0.61)	– (2.71)	– (1.08)
<i>cnc4425</i>	+ (NA)	+ (NA)	+ (NA)
<i>cnc4727</i>	– (0.90)	– (5.02)	– (0.81)
<i>cnc5368</i>	– (0.95)	– (3.17)	– (1.16)
<i>cnc5998.1</i>	– (1.10)	– (NA)	– (1.04)
<i>cnc5998.2</i>	+ (NA)	+ (NA)	+ (NA)
<i>cnc6624</i>	– (1.13)	– (1.07)	+ (1.45)
<i>cnc7476</i>	+ (26.40)	+ (19.21)	+ (17.98)

^a *cnc3005(5-7)* (spanning three predicted ncRNAs in *SCO3005*), *cnc3005.5*, *cnc4425*, and *cnc5998.2* could not be compared to the background since unspecific products were observed, but a clear peak was detected in the real sample, indicating the presence of a transcript. *cnc4425* and *cnc6624* may have been a result from a readthrough of a convergent mRNA corresponding to *SCO4424* and *SCO6623*, respectively. All other ncRNAs that were confirmed are most likely from individual transcripts.

^b qRT-PCR was used to detect the antisense RNAs in three strains of *S. coelicolor* (M145 [wild type], M751, and M752). A plus sign (+) indicates confirmed antisense ncRNAs, and a minus sign (–) indicates those not confirmed. NA, not available due to a noisy dissociation curve. The differences of qRT-PCR signal strengths compared to background are indicated in parentheses. The ncRNA numbers correspond to the SCO numbers of the target gene. *cnc2966* was the negative control.

ncRNA in *S. coelicolor* and to show that it has a regulatory effect on cellular physiology: the overexpression of the ncRNA of the GSI gene yielded surprising growth and antibiotic production defects. This suggests that the abundant ncRNAs we identified may also play a crucial physiological role in *Streptomyces*. A decrease in growth by overexpression of artificial GSI antisense ncRNAs has been observed in *Mycobacterium tuberculosis* (8). The decrease in glutamine synthase activity led to the reduced poly-L-glutamate/glutamine in the cell wall, which in turn affects mycobacterial growth. Poly-L-glutamate has been recently identified from *Streptomyces* cell wall teichoic acids (16), and this may also be affected by GS activity, explaining our observed decrease in growth. Although the effect of decreased growth and attenuated antibiotic production obviously are not biotechnologically useful traits, we believe that our data demonstrate the general possibility of gene knock-down of essential and or relevant target genes by antisense ncRNA in bacteria that was thus far restricted to eukaryotes.

cis-Encoded ncRNAs in bacteria have been identified previously in a few cases (3). These were mostly restricted to plasmids, and only a handful were observed in the bacterial chromosome. For such *cis*-encoded ncRNAs, RNase III degrades the double-stranded RNA complex formed by binding to the target sequence (3). Although the genome of *S. coelicolor* does not contain obvious homologues of Hfq, which is thought to be required for the processing of *trans*-encoded ncRNA, it does contain an RNase III (*AbsB*, *SCO5572*) homologue. *AbsB* was first identified by its effect in antibiotic

production (13) and was later shown to degrade double-stranded RNA (4). Recently, the deletion of the RNase III resulted in impaired antibiotic production and altered morphological differentiation (7, 15). Another possible mechanism for the effect of the overexpressed antisense ncRNA could be that the ncRNA inhibits transcription or translation (23). *S. coelicolor* (and probably related bacteria as well) may use these specific machinery of *cis*-encoded ncRNA as a novel and general regulatory mechanism, far beyond the limited plasmid-encoded examples reported earlier.

ncRNAs in eukaryotes are far more common than in bacteria and may reflect the complex regulation needed in multicellular organisms (24). The apparent abundance of functional ncRNAs we found in *S. coelicolor* could reflect its increased regulatory needs, given its unusually large genome (similar to yeast), an ability to adapt to a wide variety of environments and nutritional sources, and a highly dynamic secondary metabolism, including the finely tuned sequence of antibiotic production pathways. These complex regulatory networks may be mediated via signaling molecules influencing ncRNA levels. Lenz et al. (10) have shown that the expression of the *trans*-encoded sRNAs *Qrr1* to *Qrr5* in *Vibrio cholerae* is mediated by Hfq and dependent on LuxO (the regulator of the *lux* operon), whose activity is controlled by the quorum-sensing autoinducers CAI-1, Hal-1, and AI-2. *Qrr1* to *Qrr5* in turn repress the expression of LuxR, the main transcriptional regulator of the quorum-sensing regulon (21). ncRNA therefore seems to play a much larger role in this group of bacteria than previously suspected.

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